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## **BPA qualitative and quantitative assessment associated with orthodontic bonding in vivo**

Kloukos, Dimitrios ; Sifakakis, Iosif ; Voutsas, Dimitra ; Doulis, Ioannis ; Eliades, George ; Katsaros, Christos ; Eliades, Theodore

**Abstract:** **OBJECTIVE:** To assess the in vivo amount of BPA released from a visible light-cured orthodontic adhesive, immediately after bracket bonding. **METHODS:** 20 orthodontic patients were recruited after obtaining informed consent. All patients received 24 orthodontic brackets in both dental arches. In Group A (11 patients), 25 ml of tap water were used for mouth rinsing, whereas in Group B (9 patients) a simulated mouth rinse formulation was used: a mixture of 20 ml de-ionized water plus 5 ml absolute ethanol. Rinsing solutions were collected before, immediately after placing the orthodontic appliances and after washing out the oral cavity and were then stored in glass tubes. Rinsing was performed in a single phase for 60s with the entire volume of each liquid. The BPA analysis was performed by gas chromatography-mass spectrometry. **RESULTS:** An increase in BPA concentration immediately after the 1st post-bonding rinse was observed, for both rinsing media, which was reduced after the 2nd post-bonding rinse. Water exhibited higher levels of BPA concentration than water/ethanol after 1st and 2nd post-bonding rinses. Two-way mixed Repeated Measures ANOVA showed that the primary null hypothesis declaring mean BPA concentration to be equal across rinsing medium and rinsing status was rejected (p-value <0.001). The main effects of the rinsing medium and status, as well as their interaction were found to be statistically significant (p-values 0.048, <0.001 and 0.011 respectively). **SIGNIFICANCE:** A significant pattern of increase of BPA concentration, followed by a decrease that reached the initial values was observed. The amount of BPA was relatively low and far below the reference limits of tolerable daily intake.

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# **BPA qualitative and quantitative assessment associated with orthodontic bonding in vivo**

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## **ABSTRACT**

**Objective:** To assess the *in vivo* amount of BPA released from a visible light-cured orthodontic adhesive, immediately after bracket bonding.

**Methods:** 20 volunteers were recruited after obtaining informed consent. All patients received 24 orthodontic brackets in both dental arches. In Group A (11 patients), 25 ml of tap water were used for mouth rinsing, whereas in Group B (9 patients) a simulated mouth rinse formulation was used: a mixture of 20 ml de-ionized water plus 5 ml absolute ethanol. Rinsing solutions were collected before, immediately after placing the orthodontic appliances and after washing out the oral cavity and were then stored in glass tubes. Rinsing was performed in a single phase for 60 seconds with the entire volume of each liquid. The BPA analysis was performed by gas chromatography-mass spectrometry.

**Results:** An increase in BPA concentration immediately after the 1<sup>st</sup> post-bonding rinse was observed, for both rinsing media, which was reduced after the 2<sup>nd</sup> post-bonding rinse. Water exhibited higher levels of BPA concentration than water/ethanol after 1st and 2nd post-bonding rinses. Two-way mixed Repeated Measures ANOVA showed that the primary null hypothesis declaring mean BPA concentration to be equal across rinsing medium and rinsing status was rejected (p-value < 0.001). The main effects of the rinsing medium and status, as well as their interaction were found to be statistically significant (p-values 0.048, <0.001 and 0.011 respectively).

**Significance:** A significant pattern of increase of BPA concentration, followed by a decrease that reached the initial values was observed. The amount of BPA was relatively low and far below the reference limits of Tolerable Daily Intake.

**Highlights:**

- Orthodontic bonding indices an increase in BPA concentration immediately after the 1<sup>st</sup> post-bonding rinse.
- Reduced BPA concentration after the 2<sup>nd</sup> post-bonding rinse.
- BPA concentrations measured after 2<sup>nd</sup> post-bonding were at the pre-bonding levels.

## INTRODUCTION

Resin-based dental materials may induce several undesirable side effects in the oral environment, including localized and systemic reactions, mainly due to release of reactive species like residual monomers, catalysts, oxidation byproducts etc [1-4]. In orthodontics, adhesives have received limited attention relative to other orthodontic materials, as apparent in the pertinent literature [5-7].

The majority of orthodontic resinous materials are derived from Bisphenol-A (BPA). The BPA configuration assembles a bulk, stiff chain that provides low susceptibility to biodegradation as well as significant strength and rigidity in BPA based dimethacrylate polymers based on monomers like Bisphenol-A glycidyl dimethacrylate (BisGMA), its ethoxylated analogue (BisEDMA), Bisphenol-A dimethacrylate (BisDMA) and urethane-modified BisGMA [8]. Although BPA is not used as a raw material in dental resin composites, it is likely to be present as an impurity from the chemical synthesis procedure [9,10].

The unique biologic effects of BPA arise at ranges within the levels of the detection threshold for a majority of analytical techniques and show a non-monotonic curve pattern on tissues, characterized by intense reactivity at low levels and no response at very high ones [11]. This model of action originates from natural human hormones, such as 17 $\beta$ -estradiol, which can generate effects at concentrations markedly lower than those required to block the specific receptors. BPA and BPA derivatives, increase the levels of reactive oxygen species [12, 13], which are known mediators of signaling cascades under physiological conditions. Elevated levels of such compounds can disrupt the cellular redox equilibrium, causing oxidative DNA damage and apoptosis in mammalian cells.

More specifically, BPA has already been shown to activate multiple cytotoxic mechanisms and induce DNA damage [14-18]. The role of BPA in the canonical apoptotic pathways has been inadequately appraised and there is limited data associating its role in mitochondrial cell death of T cell lines [19] and germ cells after UV irradiation and hydroquinone treatment [20]. Moreover, epidemiological and genetic studies have shown that BPA is an environmental estrogenic compound that can exert proliferative responses and more specifically may induce hormonal-related effects [15, 20-26].

Although the majority of the evidence on the effect of BPA derives from *in vitro* or animal studies, recent research with human tissues confirmed intense redox activity and cross-linking of the DNA in human spermatozoa [27]. In addition, epidemiologic assays have demonstrated augmented incidence of infertility treatment and an increase in the number of abnormal sperm heads among female and male workers respectively in the plastics industry [28,29].

In orthodontics, BPA dimethacrylate derivatives are mostly used for bonding brackets (bonding resins and resin composites as main adhesives) and lingual retainers, whereas BPA-polycarbonates are used for manufacturing plastic brackets. *In vitro* studies have documented the release of BPA from polycarbonate brackets [30], orthodontic adhesives [31,32] and resin composites that are frequently used for bonding lingual retainers [30]. For traditional and flowable resin composites used as lingual retainers, BPA release was confirmed *in vivo* as well [33], with the highest values in saliva measured immediately after polymerization.

The aim of the present study was to assess the likelihood of *in vivo* release of BPA from a visible light-cured orthodontic adhesive, immediately after bracket bonding, between two groups of patients using different mouth-rinsing solutions. The

primary null hypothesis was that the mean BPA concentration would not vary between study groups. An additional objective was to quantify the released BPA by gas chromatography-mass spectrometry (GC-MS).



## **MATERIALS AND METHODS**

### **Patients and setting**

Volunteers were recruited from patients undergoing orthodontic treatment in the [removed for anonymity]. The study design was approved by the ethics and research committee of the institution (Approval Number: F.076/AD.20714) and was accomplished in accordance with the guidelines of the Declaration of Helsinki. All patients signed an informed consent before commencement of the study.

Recruitment of eligible patients began in September 2013 and ended in June 2014. Volunteers with resin fillings, sealants or other resin restorations were excluded from the study. In addition, volunteers with occupation related to chronic and severe BPA exposure or in any work environment associated with plastics were excluded as well.

### **Clinical procedures**

A total of 20 patients were finally considered eligible for inclusion. All patients received fixed orthodontic appliances in the upper and lower jaw (1st molar to 1st molar of the contralateral side). For standardization purposes, one orthodontist performed all bracket bonding procedures. Metallic brackets were used in all patients (In-Ovation R, Dentsply GAC, Milford, DE, USA). The materials used for the bonding procedure are listed in Table 1. The bonding procedure was performed as follows: The buccal surfaces of the teeth were cleaned with a fluoride-free prophylaxis pumice, rinsed with water and then acid-etched with the gel etchant for 15 s. Following 5 s water rinsing and 5 s air-drying, the primer was applied on the acid-etched surface in a thin layer. Then, the orthodontic adhesive was applied onto the

bracket base, the bracket was pressed against the primed enamel, flash resin was carefully removed with a sharp explorer and finally light-cured by using a LED curing unit emitting 1400 mW/cm<sup>2</sup> light intensity at 395-480 nm range (Valo, Ultradent Products, Inc, S. Jordan, UT, USA). Light-curing was performed by irradiating the occlusal and gingival margins of the adhesive, for 5 s each.

The patients were randomly classified in two groups (A=11, B=9). Simple, computer-based, randomization was implemented. To evaluate the levels of BPA release, rinsing solutions were collected from each patient in the same appointment, at three different periods: a) before bracket bonding, b) immediately after bracket bonding (first rinse) and c) immediately after the first rinse (second rinse). In Group A, 25 ml of tap water were used for mouth rinsing, whereas in Group B a mixture of 20 ml de-ionized water plus 5 ml absolute ethanol were used. Rinsing was performed in a single phase for 60 s with the entire volume of each liquid. Only glassware was used in the process involving sample collection and storage to prevent background contamination of BPA. All samples were then refrigerated at 4°C until analysis.

## **BPA determination**

### **a) BPA extraction**

BPA was recovered from samples by employing solid phase extraction (SPE) cartridges (OASIS HLB, 6cc/200mg, 30µm particle size, Waters Corp., Milford, MA, USA). The cartridges were placed on a vacuum manifold and conditioned sequentially with acetone, methanol and Milli-Q water (Merc Millipore, Billerica, MA, USA). The sample was percolated through the cartridges at a flow rate 5ml/min. Then, the cartridges were dried under nitrogen and BPA was eluted with acetone. The eluates were evaporated up to 0.5ml volume in a rotary evaporator and then up to dryness

under a mild stream of nitrogen. The extracted compound was submitted to derivatization by adding 100  $\mu$ L of N<sub>2</sub>O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Fluka, Buchs, Switzerland) at 70°C for 30 min. Surrogate standard deuterated Bisphenol A (BPA-d16, Aldrich, Dorset, UK) was added in all samples before extraction as well as in standard solutions used for calibration.

#### **b) Analytical determination of BPA**

The BPA analysis was performed by gas chromatography-mass spectrometry [35] employing a gas-chromatograph (Trace GC Ultra, Thermo Finnigan Electron Corporation, Waltham, MA, USA) coupled with an ion trap mass spectrometer (Polaris Q, Thermo Finnigan) and an autosampler (AI 3000, Thermo Finnigan). A 5% diphenyl-95% dimethylpolysiloxane capillary column of 30 m length, 0.25 mm internal diameter and 0.25  $\mu$ m film thickness (Rtx-5MS Crossbond, Thames Restek Ltd., Bucks, UK) was used with He carrier gas at a flow rate of 1.5 ml/min. The column temperature program was set as follows: Initial T=80°C for 1 min, increase up to T=150°C at 20°C/min rate, further increase up to T=280°C at 10°C/min rate, which was maintained for 2 min. The temperature of the injector was 280°C, the ion source 200°C and the transfer line 300°C, respectively.

Mass spectra were obtained using electron impact ionization (70 eV). The identification of BPA and BPA-d16 was based on relative retention times, the presence of target ions ( $m/z$  357.2 and 358.2 for BPA and 368.3 and 369.3 for BPA- d16) and their relative abundance. BPA was quantified by the relative response factor to the surrogate internal standard BPA-d16. The  $m/z$  357.2 and 368.3 were used for quantitation of BPB and BPA16, based on relative response factor. A linear fit with high correlation coefficient (0.998) was obtained for the working standards. The

instrumental repeatability was 2.3% and the detection limit was 0.5ng/μL. The recovery of BPA ranged from 97-104%.

## **Statistical Analysis**

Descriptive statistics (mean and standard deviation) of the BPA concentration by rinsing medium and status were calculated. The rinsing medium (water, water/ethanol) and the rinsing status (before bracket bonding, after the 1st rinse and following the 2nd rinse) divided the dataset into six subgroups. The primary null hypothesis was that the mean BPA concentration did not vary between these six subgroups. This null hypothesis was tested by using two-way mixed repeated measures analysis of variance (2-way mixed RM ANOVA) with the BPA concentration as the dependent variable. The fixed portion of the model included rinsing medium, rinsing status and their interaction. The random portion of the model was the rinsing medium. Post-hoc comparisons were performed by Sidak's correction. The level of statistical significance was set to 95% ( $\alpha = 0.05$ ). All analyses were conducted using Stata 13.0/SE software (StataCorp LP, College Station, TX, USA).

## RESULTS

Representative GC-MS chromatograms for a sample and a control are illustrated in Figure 1 (a-d).

The descriptive statistics of BPA concentration per rinsing medium and status are presented in Table 2. An increase in BPA concentration immediately after the 1<sup>st</sup> post-bonding rinse was observed, for both rinsing media, which was reduced after the 2<sup>nd</sup> post-bonding rinse. Water exhibited higher levels of BPA concentration than water/ethanol after 1<sup>st</sup> and 2<sup>nd</sup> post-bonding rinses.

Two-way mixed Repeated measures ANOVA (Table 3) showed that the primary null hypothesis declaring mean BPA concentration to be equal across rinsing medium and rinsing status was rejected ( $p\text{-value} < 0.001$ ). The main effects of the rinsing medium and status, as well as their interaction were found to be statistically significant ( $p\text{-values}$  0.048,  $<0.001$  and 0.011 respectively).

Table 4 reports the results of the post-hoc analysis employing Sidak's correction ( $\alpha_{\text{Sidak}} = 0.0056$ ). For the water rinsing group, a statistically significant ( $p\text{-value} < 0.001 < \alpha_{\text{Sidak}}$ ) increase in BPA concentration from pre-bonding to 1<sup>st</sup> post-bonding measurement was observed, estimated at 147 ng/l (unadjusted 95% CI = 99.42, 193.12), followed by a statistically significant ( $p\text{-value} < 0.001 < \alpha_{\text{Sidak}}$ ) decrease, estimated at 96.09 ng/l (unadjusted 95% CI = -142.94, -49.24) from 1<sup>st</sup> post-bonding to 2<sup>nd</sup> post-bonding measurement. The respective difference between 2<sup>nd</sup> post-bonding and pre-bonding measurement, estimated at 50.18 ng/l (unadjusted 95% CI = 3.33, 97.03), was not statistically significant ( $p\text{-value} = 0.036 > \alpha_{\text{Sidak}}$ ). Consequently in the water rinsing group, BPA at the final (2<sup>nd</sup> post-bonding) measurement reached the corresponding pre-bonding levels.

For the water /ethanol rinsing group, a statistically non-significant ( $p\text{-value} = 0.132 > \alpha_{\text{Sidak}}$ ) increase in BPA concentration from pre-bonding to 1<sup>st</sup> post-bonding measurement was observed, estimated at 39.33 ng/l (unadjusted 95% CI = -12.46, 91.13), followed also by a statistically non-significant ( $p\text{-value} = 0.294 > \alpha_{\text{Sidak}}$ ) decrease, estimated at 66.56 ng/l (unadjusted 95% CI = -79.01, 24.57), from 1<sup>st</sup> post-bonding to 2<sup>nd</sup> post-bonding measurement. The respective difference between 2<sup>nd</sup> post-bonding and pre-bonding measurement, estimated at -27.22 ng/l (unadjusted 95% CI = -79.01, 24.57), was also statistically non-significant ( $p\text{-value} = 0.013 > \alpha_{\text{Sidak}}$ ). Therefore, in the water/ethanol rinsing group, the BPA concentrations measured after 1<sup>st</sup> and 2<sup>nd</sup> post-bonding were at the pre-bonding levels.

The differences of BPA concentration between the two rinsing media showed no statistically significant difference in pre-bonding and 2<sup>nd</sup> post-bonding measurements ( $p\text{-value} = 0.355$  and  $p\text{-value} = 0.051$  respectively), estimated at -24.60 ng/l (unadjusted 95% CI = -76.31, 27.09) and 52.80 ng/l (unadjusted 95% CI = 1.10, 104.50) respectively. At 1<sup>st</sup> post-bonding, BPA concentration was higher in the water rinsing group by 82.33 ng/l (unadjusted 95% CI = 30.63, 134.03), which was a statistically significant difference ( $p\text{-value} = 0.003 < \alpha_{\text{Sidak}}$ ).

Figure 2 summarizes the results of the statistical analysis per rinsing medium and status.

## DISCUSSION

The results of the present study showed a statistically significant BPA release in the water group, following the 1st post-bonding rinsing, with all other differences between rinsing groups and among rinsing status being non-significant. Therefore, the null hypothesis should be rejected for this group.

BPA has shown potential estrogenicity in a significant number of studies [35] and is described as an endocrine disruptor chemical, capable of activating the human estrogen receptor, but at a capacity of 1000–5000 times less than the endogenous 17- $\beta$  oestradiol [36]. Concerning the BPA safety issues, the European Food Safety Authority announced an initial risk assessment, based on a tolerable daily intake (TDI) of 50  $\mu\text{g}/\text{kg}$  body weight/day [37]. Several scientists arguably disputed the use of TDI for risk assessments on endocrine disruptor chemicals, suggesting that their effects are observed at very low doses, non-monotonic dose–response curves, as well as on effects occurring from very specific windows of exposure [38].

The concerns on BPA exposure are significant for children, since a pediatric review indicated that psychosocial health in different child ages might be influenced after exposure to dental composite resins based on BPA derivatives. Higher levels of anxiety, depression, social stress and interpersonal- relation problems in children were reported, probably due to increased levels and duration of exposure to dental composites [39]. Nevertheless, a recent study by the same authors failed to reach the same conclusions for flowable restoratives and sealants, which are much more prone to intraoral degradation than conventional resin composites, due to their reduced or minimal filler content [40]. Apparently such correlations are dependent on many uncontrolled variables.

The amount of BPA released from resin composites has been assessed in many studies [10]. Although much lower than the TDI, the 24-h release of BPA from dental materials was pertinent in patients with multiple or large restorations, representing a significant source of BPA in such patients [10].

The current study evaluated the amount of BPA released immediately after bonding fixed orthodontic appliances with adhesives based on BPA derivatives, since in vivo data confirmed that the highest release from resin composites used as fixed retainer adhesives was observed during the early post-bonding period [33]. Gas chromatography and mass-spectrometry was used as the main analytical method, since BPA is volatile and thermal stable to be detected by gas chromatography and traced at low levels provided by mass-spectrometry [41].

The highest amount observed in the water group after the 1st post-bonding rinse should be attributed to the oxygen inhibited layer of the adhesives set in air. After removal of this layer, the BPA concentration was reduced to the level of the pre-bonding control. For the cases treated with metallic brackets as in the current experiment, the thickness of the adhesive resin exposed to the bracket margins may range from 150-250  $\mu\text{m}$  [42] dependent on the bracket base design and resin viscosity. Although small in thickness, the total surface area of the resin exposed may considerably increase for a full-arch bonding. The oxygen inhibited layer composed of unreacted monomer species can dissolve in saliva or rinsing media and released intraorally. The concurrent release of BPA depends on the purity of the monomers used in the adhesive manufacturing process [43], since the time span used in the present study was very short to allow for bulk release of residual monomers or degradation products. Therefore, removal of the oxygen inhibited layer immediately after bonding should be considered mandatory. Pumice prophylaxis of the cured



surfaces exposed to air has been shown to reduce BPA release in sealants and resin composites used as orthodontic retainers [33, 44], where large material areas are exposed to air during setting. For bracket bonding, an intense water rinsing of the brackets' periphery assisted by a strong suction unit may remove efficiently the inhibited zone and consequently exposure to BPA.

The use of water/ethanol (4:1) rinsing medium was selected to simulate ethanol-based mouthrinses. Since the BPA solubility in ethanol is almost 100 times greater than in water [45], it was expected that the water/ethanol rinsing solution could remove more easily the oxygen inhibited layer. Nevertheless, no statistically significant differences were found among the rinsing modes. This might be assigned to the higher solubility of BPA in water, the reduced exposure of the material to the rinsing solution which was limited to the bracket-enamel margins, and the potential reduced rinsing action of patients in the ethanolic solutions because of their taste.

The results of BPA concentration after the pre-bonding rinse indicate the extent of environmental exposure of the saliva of each volunteer and of the rinsing water. The pooled values ranged from 69-290 ng/l are within the limits previously reported for saliva and water.

The results of the present study indicated that the orthodontic adhesives tested immediately released BPA immediately after setting, but in much lower levels compared with the TDI limits. Nevertheless, due to the controversy regarding the safe level of BPA exposure [46], it appears best not to expose patients to BPA. Currently several orthodontic adhesives have been introduced free of BPA components, mainly based on aliphatic dimethacrylates. Nevertheless, the absence of the BPA structure does not essentially imply the lack of estrogenic activity, since several BPA-free

chemicals used as replacement for BPA containing resins have been shown to trigger an estrogenic effect [47].

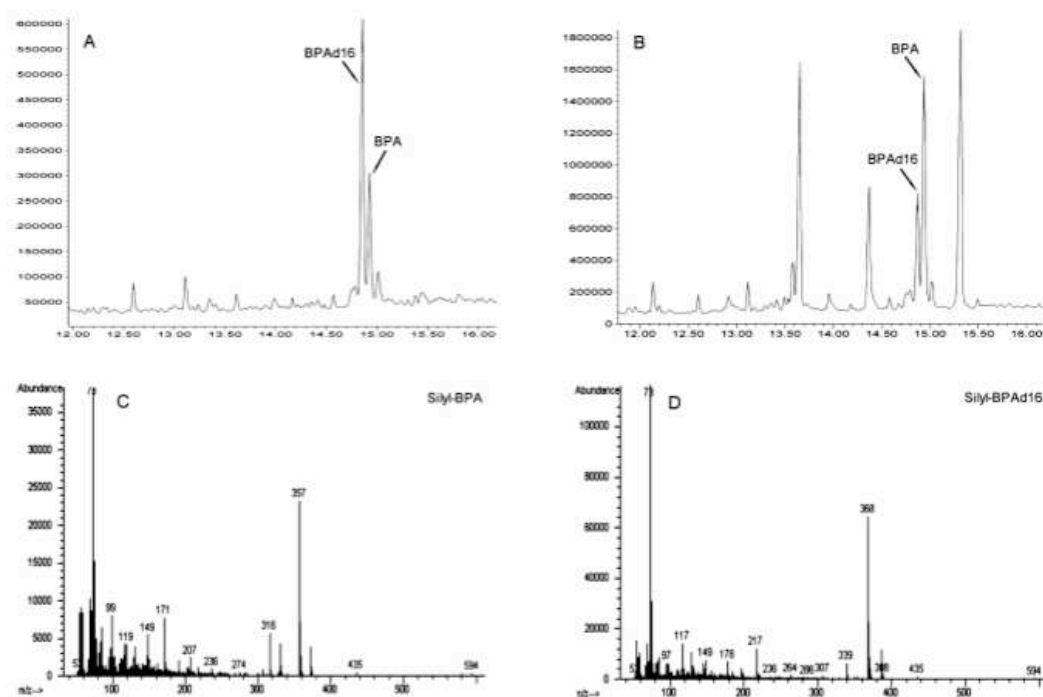
### **Significance**

A significant pattern of increase of BPA concentration, followed by a decrease that reached the initial values was observed. The amount of BPA was relatively low and far below the reference limits of Tolerable Daily Intake. Clinically, rinsing with water after debonding may be employed to reduce the BPA presence.

## FIGURE LEGENDS

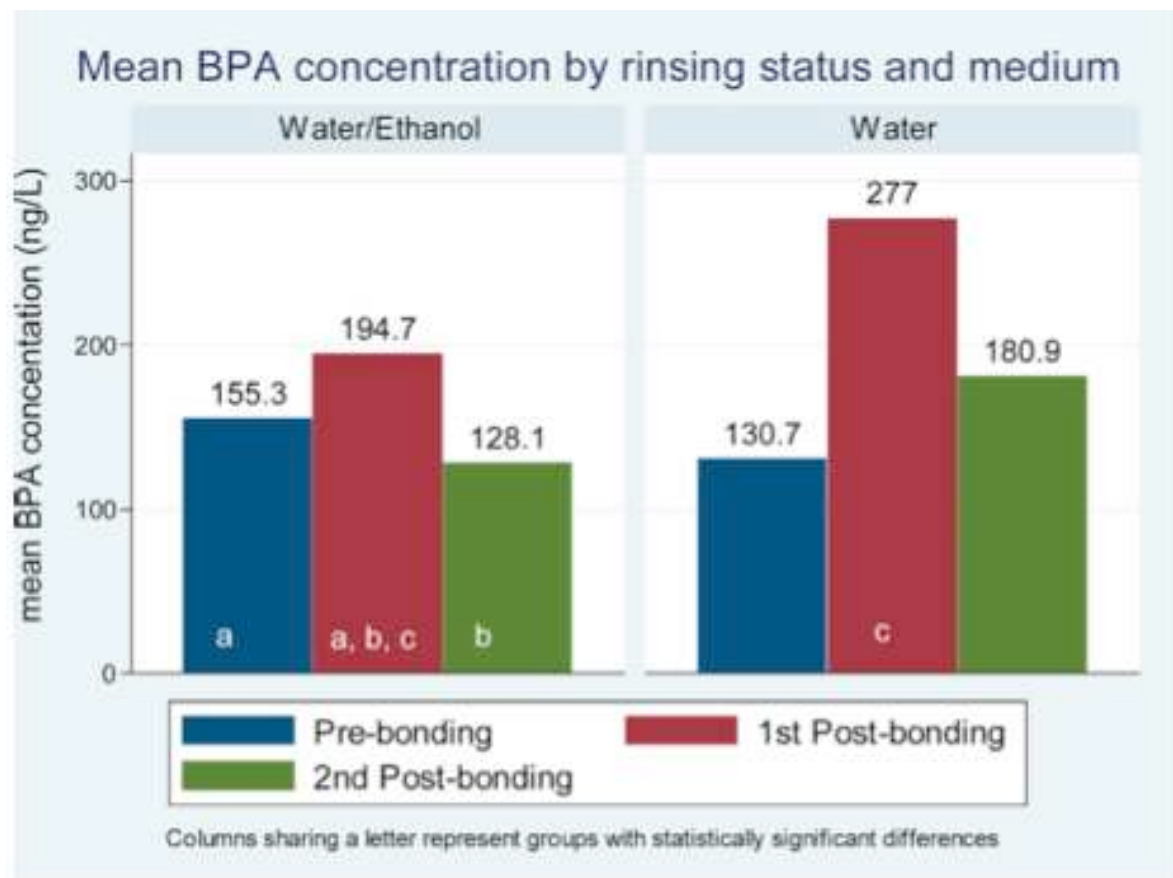
### Figure 1 (a-d)

GC-MS chromatograms for a sample and a control For standard (A) and samples (B). Mass fragments of trimethyl-silylated derivatives of BPA (C) and BPAd16 (D) are also shown.



**Figure 2**

Comparisons of mean BPA concentrations by rinsing medium and status.



**Table 1.**

The composition of the products tested, according to the manufacturer's information.

PRODUCT	COMPOSITION (wt% range)	MANUFACTURER
Scotchbond Etchant	Water (55-65), Phosphoric acid (30-40), Synthetic amorphous silica (5-10)	3M Unitek, Monrovia CA, USA
Transbond MIP Primer	Resin: Bisphenol-A diglycidyl ether dimethacrylate (15-25). 2-Hydroxyethyl methacrylate (10-20) 2-Hydroxy-1,3-dimethacryloxypropane (5-15) Copolymer of itaconic and acrylic acid (5-15) Diurethane dimethacrylate (1-10) Solvents: Ethyl alcohol (30-40) Water (1-10)	3M Unitek, Monrovia CA, USA
Transbond XT Adhesive	Resin: Bisphenol-A diglycidyl ether dimethacrylate (10-20) Bisphenol-A bis(2-hydroxyethyl ether) dimethacrylate (5-10) Fillers: Silane treated quartz (70-80), Silane treated silica (<2), Catalysts: Diphenyl iodonium hexafluorophosphate (<0.2)	3M Unitek, Monrovia CA, USA

**Table 2.**

Descriptive statistics (mean, standard deviation) of BPA concentration by rinsing medium and status.

	RINSING MEDIUM	
	Water/Ethanol (N=9)	Water (N=11)
RINSING STATUS	BPA (ng/l), mean (SD)	
Pre-bonding	155.33(31.32)	130.73(72.60)
1st post-bonding	194.67(55.10)	277.00(72.18)
2nd post-bonding	128.11(32.17)	180.91(63.84)

**Table 3.**

Two-way mixed Repeated Measures ANOVA. Main findings.

	<b>F-statistic</b>	<b>Degrees of freedom</b>	<b>P-value</b>
<b>MODEL</b>	3.59	23	0.000 <sup>a</sup>
Main effects			
Rinsing medium	4.516	1	0.048 <sup>b</sup>
Rinsing status	17.269	2	0.000 <sup>a</sup>
Interaction	5.144	2	0.011 <sup>b</sup>

<sup>a</sup>Statistically significant (a = 0.001).<sup>b</sup>Statistically significant (a = 0.05).**Table 4.**

Two-way mixed Repeated Measures ANOVA. Post-hoc pairwise comparisons with Sidak's correction.

<b>Pairwise comparisons</b>	<b>Estimated difference</b>	<b>Standard error</b>	<b>Unadjusted 95% CI</b>	<b>Unadjusted p-value</b>
<b>Between rinsing status and within rinsing medium</b>				
<i>A. Water</i>				
1 <sup>st</sup> Post-bonding vs Pre-bonding	146.27	23.10	(99.42, 193.12)	0.000*
2 <sup>nd</sup> Post-bonding vs Pre-bonding	50.18	23.10	(3.33, 97.03)	0.036
2 <sup>nd</sup> Post-bonding vs 1 <sup>st</sup> Post-bonding	-96.09	23.10	(-142.94, -49.24)	0.000*
<i>B. Water/Ethanol</i>				
1 <sup>st</sup> Post-bonding vs Pre-bonding	39.33	25.54	(-12.46, 91.13)	0.132
2 <sup>nd</sup> Post-bonding vs Pre-bonding	-27.22	25.54	(-79.01, 24.57)	0.294
2 <sup>nd</sup> Post-bonding vs 1 <sup>st</sup> Post-bonding	-66.56	25.54	(-118.35, -14.76)	0.013
<b>Between rinsing medium and within rinsing status</b>				
A. Pre-bonding	-24.60	26.38	(-76.31, 27.09)	0.351
B. 1 <sup>st</sup> Post-bonding	82.33	26.38	(30.63, 134.03)	0.002*
C. 2 <sup>nd</sup> Post-bonding	52.80	26.38	(1.10, 104.50)	0.045

\*Statistically significant (a<sub>Sidak</sub> = 0.0056).

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